

REMARKS/ARGUMENTS

Claims 1-39 are currently pending. Applicant has canceled claims 1-39, and presented new claims 40-80, to correct a numbering problem where claim 28 was listed twice. New claims 40-80 are directed to the same subject matter, and therefore, no new matter has been added by these amendments. Applicant has indicated in the claim status indicator, those renumbered claims which have also been amended since the last Office Action as “(New, amended)” to aid the Examiner. Claims 5-8, 10-18, and 23-25, now corresponding to new claims 44-47, 49-57, and 62-64 were withdrawn from consideration as non-elected subject matter. No new matter has been added by these amendments.

Discussion of Claim Objections

The Examiner objected to claims 1, 26, 29, 31 and 36-38 because of certain informalities. Applicant has replaced the term “an” with “and” in claims 40 and 78, which correspond to previous claims 1 and 38. Applicant has written out the term “bovine corneal endothelial cell-extracellular matrix” BCE-ECM in claim 63, which is the first instance of the term in the claims, and corresponds to original claim 24. Claims 71 and 74 now include the term “A,” and correspond to original claims 29 and 31. Claims 76 and 77, corresponding to original claims 36 and 37, were amended to be independent. Applicants respectfully request that the claim objections be withdrawn as moot, in view of Applicant’s new claims. No new matter has been added by these amendments.

Discussion of Rejections under 35 U.S.C. §112, second paragraph

The Examiner rejected claims 1-4, 9, 19-22, 27-35, 38 and 39 under 35 U.S.C. §112, second paragraph, as indefinite. Regarding claim 1, corresponding to new claim 40, Applicant has amended the claim to delete the terms “high-quality” and “surface” from the claim. Applicant submits the rejection of claim 40 is rendered moot, and respectfully request withdrawal of this rejection.

Regarding claims 9, 21, and 27 (now claims 50, 56 and 60), the term “RGDS” does indicate the tetrapeptide Arg-Gly-Asp-Ser. This molecule and term is well known in the cell culture and cell adhesion molecule arts as a recognition sequence for cell surface proteins such as integrins. See for example, U.S. Patent No. 7,517,954. RGDS is also well known abbreviation and has been used for over twenty years. See, for example, McCarthy, J., et al., J. Cell Biol., 110: 777-787 (1990) (Attachment A). Thus, Applicant submits that the term RGDS, at the time Applicant’s application was filed, would have been understood by those of ordinary skill in the art, and therefore, the metes and bounds of the claim would not be considered by those of ordinary skill in the art to be indefinite. Applicant respectfully requests withdrawal of this rejection.

The Examiner rejected claims 19-22 (now 58-61), as being indefinite. The Examiner alleges that claim 19 (58) omits the word “cell” in the phrase “...neuronal cell growth coated with Diamond-like-Carbon...” Applicant submits that the term “neuronal” does not render claim 19 (58) indefinite, because the term would be understood by those of ordinary skill in the cell biology arts, to refer to neuronal cells, or “neurons,” or “neural cells” as referred to earlier in the claim, which are both commonly understood, and acceptable terms in the art. However, solely for purposes of advancing prosecution, and without acquiescence to the Examiner’s position, Applicant has amended claim 19 (58) to insert the term “cell” as suggested by the Examiner. Applicant respectfully requests withdrawal of this rejection as moot.

The Examiner rejected claims 20 and 27 (now 59 and 66), as indefinite for reciting the term “May Polymer.” This term was an internal name given by Applicant for a biopolymer made of either chitosan or sodium alginate, or a mixture thereof. Applicant has amended claims 20 and 27 (59, 66) to delete the term “May Polymer” and insert the phrase “comprised of chitosan and/or sodium alginate.” Applicant submits that this amendment is fully supported in Applicant’s specification at paragraph [0011], thus no new matter has been added by this amendment. In view of this amendment, Applicant respectfully requests withdrawal of this rejection as moot.

The Examiner also rejected claims 21 and 28(i) (now 60, 67), as being dependent to claims 20 and 27, and further limiting the term “May polymer.”

Applicant submits that in view of Applicant's amendment of claims 59 and 66, this rejection is rendered moot. In addition, the Examiner stated that the phrase "into said unit" in both claims, lacked antecedent basis. Applicant has amended claims 60 and 67 by deleting the phrase "into said unit," and inserting the phrase "onto the growth medium," to further refine and clarify that which Applicant regards as the claimed invention. Support for this amendment can be found throughout the specification, and for example, at paragraphs [0010], [0011], [0018] and [0019]. Applicant therefore respectfully requests withdrawal of this rejection.

The Examiner rejected claim 29 (now, 69) as indefinite, because the Examiner considered the phrase "comprising said apparatus" as being circular and unclear. Applicant has amended claim 69, by deletion of the phrase "comprising said" and inserting "the," to recite that the apparatus has an inside and an outside. With regard to claim 30, the Examiner also indicated that claim 30 was indefinite because the recited species of "slides" did not appear to correlate with claim 29 as slides do not have an inside and outside surface. Applicants respectfully disagree.

Claim 29 states that "...wherein the inside surface is the surface in contact with cells and cellular media and the inside surface of said apparatus is coated with a film of Diamond-like-Carbon." While not wishing to be bound to any particular embodiment, Applicant submits that one of ordinary skill in the art would understand that a slide for growing cells can comprise an inner surface where the cells are grown, and can also comprise a chamber which can be surrounded by walls in which a quantity of growth media can be contained on top of the cells growing on the inside surface in the chamber. There may also be a cover that sits on top the cell chamber. Alternatively, the slide can be a removable glass, or plastic insert, which is placed in a dish. In either embodiment, the "inside surface" of the slide would be understood by those of ordinary skill to be the side that the cells are growing on, and the outside would be the surfaces the cells are not growing on. Applicant therefore respectfully request withdrawal of this rejection.

The Examiner has rejected claim 31 (now 71) as indefinite, because the Examiner states the claim is directed to both an apparatus and a method of manufacture. Applicant has amended the claim to recited that the apparatus has an

inside surface which is in contact with the cells having a coating of Diamond-like-Carbon layered over at least one other coating. This amendment is fully supported by the specification. Applicant submits that in view of this amendment, the rejection of claims 31-33 are rendered moot. Applicant, therefore, respectfully requests withdrawal of this rejection.

Claim 35 (now 75) was rejected for lacking antecedent basis for the term “the inside surface of said apparatus.” Applicant has amended claims 74 and 75 to further clarify and refine that which Applicant regards as the invention. In particular, Applicant has inserted language into claim 74 which more directly conforms to a claims to a method of applying a coating to the inside surface of a laboratory apparatus. Claim 75 was amended to reflect these amendments to claim 74. As such, Applicant submits the rejection of claim 75 is rendered moot, and respectfully request withdrawal of this rejection.

Claim 38 and 39 (now 78 and 79) were rejected as being indefinite, for substantially the same reasons as the rejection of claim 1 above. Applicant has amended the claim to delete the terms “high-quality” and “surface” from the claim. As such, Applicant submits the rejection of claims 78 and 79 are rendered moot, and respectfully request withdrawal of this rejection.

Claim 39 (79) was also rejected as being indefinite, because the Examiner alleges that is unclear what the scope of the claim is. The Examiner argues that the term “an acrylic polymer and its derivatives or copolymers” is a genus, but that Applicant then recites other species without identifying whether these species are examples or members of the genus.

Applicant has amended claim 79 to recite that the biopolymer is a biodegradable acrylic polymer. Support for the amendment can be found in Applicant’s specification, for example, at paragraphs [0011] and [0014]. Applicant has added new dependent claim 80, which is directed to the specific biopolymers originally identified in claim 79, and is in the form of a Markush group. In view of these claim amendments, Applicant submits the rejection of claims 78 and 79 are rendered moot, and respectfully request withdrawal of this rejection.

Discussion of Rejections under 35 U.S.C. §101

The Examiner rejected claims 31-33 (now 71-73) as being directed to nonstatutory subject matter, because the claim is directed to both an apparatus and a method of making an apparatus. In view of Applicant's claim amendments, Applicant submits the rejection of claims 71-73 are rendered moot, and respectfully request withdrawal of this rejection.

Discussion of Rejections under 35 U.S.C. §112, first paragraph

The Examiner rejected claim 39 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Examiner alleges that in claim 39, the claim language "acrylic polymers and its derivatives and copolymers" is overly broad. Applicant respectfully disagrees.

One of ordinary skill in the art would understand what is meant by the genus of acrylic polymers, derivatives and copolymers. Applicant has amended claim 39 to recite "biodegradable acrylic polymers." Applicant submits that the amended claim is fully supported by the specification, for example, at paragraph [0014].

Discussion of Rejections under 35 U.S.C. §102

The Examiner rejected claims 29, 30, 34 and 36 (now 69, 70, 74 and 76), under 35 U.S.C. §102(b) as being anticipated by Lu et al., Bio-Medical Materials and Engineering (1993) ("Lu et al."). The Examiner alleges that Lu et al. teach the deposition of Diamond-like-Carbon films onto cell culture dishes which reads on Applicant's claims directed to "laboratory apparatus." Lu et al. do not teach or suggest the use Diamond-like-Carbon to coat laboratory apparatus which have a biopolymer coating. Applicant respectfully disagrees.

Applicant has amended claims 69, 71, 72, 74, and 75 to reflect the fact that the apparatus will be coated with a biopolymer coating prior to being coated with a film of Diamond-like-Carbon. The claims also reflect that there can be at least one other

coating with the biopolymer, prior to coating with a film of Diamond-like-Carbon. These amendments are fully supported by Applicant's specification, including, for example, paragraphs [0014] and [0020].

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." Verdegaal Bros. v. Union Oil Co. of California, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). With regard to claims 69, 71, 72, 74, and 75 as currently amended, Lu et al. do not teach that the apparatus will be coated with a biopolymer coating prior to being coated with a film of Diamond-like-Carbon. As such, Lu et al. do not teach each and every element of amended claims 69, 71, 72, 74 and 75, and therefore these claims cannot be anticipated under 35 U.S.C. § 102(b). Applicants respectfully request withdrawal of the rejection.

The Examiner also rejected claims 31-37 (now 71-77), under 35 U.S.C. §102(b), as being anticipated by Ignatius et al., J. Biomed. Res. (1998) ("Ignatius et al."). The Examiner alleges that Ignatius et al. disclose coating glass coverslips with Diamond-like-Carbon, and then were further coated with laminin, fibronectin or collagen. According to the Examiner, laminin is a component of ECM and therefore Applicant's claims read on the disclosure of Ignatius et al. Applicant respectfully disagrees.

Ignatius et al. teach that glass coverslips were washed in acid or alcohol, then coated with Diamond-like-Carbon, or metal. After that coating, they are coated with poly-L-lysine for three hours, and then washed and dried. For experiments containing laminin or fibronectin or collagen, these were then separately added later (See, "Substrate Preparation" in Ignatius et al. at pages 265-266). This is not how Applicant's apparatus is made, nor is the final resulting coating or apparatus the same.

Applicant applies the biopolymer to the apparatus first, followed, optionally by at least one other coating, which could be various attachment factors or BCE-ECM. A layer of Diamond-like-Carbon is then applied to the other layer(s). Thus, the composition of Applicant's claimed coatings are not the same as those taught in Ignatius et al., because the layers are in the reverse order, and all of the slides taught

in Ignatius et al. are coated with poly-L-lysine after the Diamond-like-Carbon film is applied, and before any other coating. There is no use of poly-L-lysine in Applicant's claimed invention.

In addition, Applicant submits that laminin cannot be considered as the same composition as BCE-ECM. Laminin, as applied, is a well-known and characterized as a large trimeric glycoprotein that contains an α -chain, a β -chain and a γ -chain, and is found in multiple genetic variants. Fibronectin is also a well-known, high-molecular weight extracellular matrix glycoprotein that binds to membrane-spanning receptor proteins called integrins. ECM (or BCE-ECM), however, is not a single protein, but a collection of various proteins and other compounds (heparin, chondroitin, keratin, hyaluronic acid, various collagens, elastin) as well as fibronectin and laminin, secreted by endothelial and other cells. It is known by those of ordinary skill in the cell culture arts that ECM varies in its composition based on the cell types and species which make it. As such, the teachings of Ignatius et al. do not teach all of the elements of BCE-ECM coatings in Applicant's claims, and therefore it cannot anticipate Applicant's claims.

Discussion of Rejections under 35 U.S.C. §103

The Examiner has rejected claims 1-4, 9, 19, 26, 38 and 39 (now 40-43, 48, 58, 63, and 78-80), under 35 U.S.C. §103(a), as obvious over Ignatius et al., in view of Lu et al. According to the Examiner, Ignatius et al. teaches the coating of glass coverslips with Diamond-like-Carbon and laminin to support growth and attachment of neuronal cells. However, Ignatius et al. do not teach the use of polymers to grow cells on. Lu et al. is provided by the Examiner to teach that cells are routinely grown on plastic surfaces, including polycarbonate, polyethylene, and polyurethane, and that such surfaces can be coated with Diamond-like-Carbon. Thus, the Examiner argues that it would have been obvious, to one of ordinary skill in the art, at the time Applicant's invention was made, to substitute the synthetic biopolymers taught in Lu et al., for the glass coverslips taught in Ignatius et al. to arrive at Applicant's claimed invention. Applicants respectfully disagree.

As stated in Applicant's remarks regarding Ignatius et al. above, the composition of Applicant's claimed coatings are not the same from what is taught in Ignatius et al., because the layers are in the reverse order, and all of the slides taught in Ignatius et al. are coated with poly-L-lysine after the Diamond-like-Carbon film is applied and before any other coating. Furthermore, the use of laminin or fibronectin individually, cannot be considered by those of ordinary skill, to be the same composition as BCE-ECM.

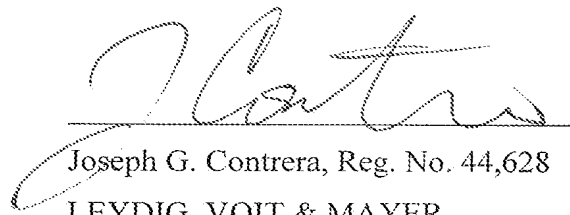
Moreover, these deficiencies are not cured by Lu et al. Applicant submits that Lu et al. do not teach biopolymers as disclosed in Applicant's claimed invention. At paragraphs [0011], [0014] of the instant specification, Applicant teaches various examples of biopolymers suitable for use in the claimed invention. It would be clear to one of ordinary skill in the art, when reading Applicant's specification, that Applicant's claimed biopolymers are not in the same chemical class of compositions as the plastics and polymers used in the tissue culture ware taught in Lu et al. For example, the examples of biopolymers in Applicant's specification are all biodegradable and water-soluble. On the other hand, polycarbonate, polyethylene and polyurethane, and other well-known plastics, as taught in Lu et al., are not biodegradable, nor water-soluble. Therefore, one of ordinary skill in the art would understand that polycarbonate, polyethylene and polyurethane, and other well-known plastics, as taught in Lu et al., cannot be considered by those of ordinary skill in the cell culture arts, as mere substitutes for the biopolymers claimed by Applicant.

To establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). In view of the foregoing, Applicant submits that the Examiner has failed to establish a *prima facie* case of obviousness with regard to claims 40-43, 48, 58, and 78-80, because the combination of teachings of Ignatius et al., in view of Lu et al. does not teach each and every claim limitation of Applicant's claimed invention. Applicant respectfully requests withdrawal of this rejection.

Conclusion

Applicant respectfully submits that the patent application is now in condition for examination and allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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RGD-independent Cell Adhesion to the Carboxy-terminal Heparin-binding Fragment of Fibronectin Involves Heparin-dependent and -independent Activities

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Abstract. Cell adhesion to extracellular matrix components such as fibronectin has a complex basis, involving multiple determinants on the molecule that react with discrete cell surface macromolecules. Our previous results have demonstrated that normal and transformed cells adhere and spread on a 33-kD heparin binding fragment that originates from the carboxy-terminal end of particular isoforms (A-chains) of human fibronectin. This fragment promotes melanoma adhesion and spreading in an arginyl-glycyl-aspartyl-serine (RGDS) independent manner, suggesting that cell adhesion to this region of fibronectin is independent of the typical RGD/integrin-mediated binding. Two synthetic peptides from this region of fibronectin were recently identified that bound [³H]heparin in a solid-phase assay and promoted the adhesion and spreading of melanoma cells (McCarthy, J. B., M. K. Chelberg, D. J. Mickelson, and L. T. Furcht. 1988. *Biochemistry*. 27:1380-1388). The current studies further define the cell adhesion and heparin binding properties of one of these synthetic peptides. This peptide, termed peptide I, has the sequence YEKPGSP-PREVVPRPRPGV and represents residues 1906-1924 of human plasma fibronectin. In addition to promoting

RGD-independent melanoma adhesion and spreading in a concentration-dependent manner, this peptide significantly inhibited cell adhesion to the 33-kD fragment or intact fibronectin. Polyclonal antibodies generated against peptide I also significantly inhibited cell adhesion to the peptide, to the 33-kD fragment, but had minimal effect on melanoma adhesion to fibronectin. Anti-peptide I antibodies also partially inhibited [³H]heparin binding to fibronectin, suggesting that peptide I represents a major heparin binding domain on the intact molecule. The cell adhesion activity of another peptide from the 33-kD fragment, termed CSI (Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1987. *J. Biol. Chem.*, 262:6886-6892) was contrasted with peptide I. Whereas both peptides promoted RGD-independent cell adhesion, peptide CSI failed to bind heparin, and exogenous peptide CSI failed to inhibit peptide I-mediated cell adhesion. The results demonstrate a role for distinct heparin-dependent and -independent cell adhesion determinants on the 33-kD fragment, neither of which are related to the RGD-dependent integrin interaction with fibronectin.

TUMOR cell adhesion to extracellular matrix components is a fundamental consideration of invasion and metastasis (Liotta et al., 1983; McCarthy et al., 1985). Over the last several years, it has been shown that the adhesion of normal and transformed cells to extracellular matrix proteins such as fibronectin has a complex molecular basis. The recognition of the arginyl-glycyl-aspartyl-serine (RGDS) sequence by cell surface integrin(s) is the best understood mechanism that cells use to adhere to fibronectin and other matrix and plasma proteins (Buck and Horwitz, 1987; Juliano, 1987; Hynes, 1987; Ruoslahti, 1988). Despite the importance of this interaction to cell adhesion, there is clear evidence indicating the involvement of other cell sur-

face macromolecules and other biologically active domains on fibronectin.

We have previously demonstrated that a 33-kD heparin-binding fragment, which originates from the carboxy-terminal region of fibronectin A-chains, is active at promoting the RGD-independent adhesion and spreading of murine melanoma and fibrosarcoma cell lines (McCarthy et al., 1986, 1988a,b). This fragment can inhibit experimental metastasis when preincubated with tumor cells *ex vivo* before tail vein injection (McCarthy et al., 1988b). Other cell types, such as neurons from the developing chicken (Rogers et al., 1987), endothelial cells (Visser et al., 1989), and lymphocytes (Liao et al., 1989; Wayner et al., 1989) also adhere to this frag-

ment. An mAb raised against the 33-kD fragment was used to localize a melanoma adhesion-promoting activity on a region of this fragment, which excluded the type IIIc connecting sequence (McCarthy et al., 1988a). This approach was complemented with the identification of two heparin-binding synthetic peptides that promoted melanoma cell adhesion (McCarthy et al., 1988a). The peptides, termed I and II, have different primary structures (YEKPGSPPREVVPRPRGV and KNNQKSEPLIGRKKT, respectively) yet they share some common features, including a low (hydrophilic) hydrophobicity index and a positive net charge. Whereas both peptides bear a net positive charge, not all positively charged peptides produced from matrix proteins (e.g., laminin) will promote melanoma cell adhesion (Charonis et al., 1988), suggesting that charge alone is not the sole explanation for the activity of such peptides. That the fibronectin peptides bind both [³H]heparin and promote cell adhesion suggests a role for cell surface heparin-like molecules (i.e., glycosaminoglycans or proteoglycans) in promoting melanoma cell adhesion to this region of fibronectin.

A synthetic peptide approach has also been used by others to identify an additional cell adhesion promoting domain within fibronectin A-chains (Humphries et al., 1987). Synthetic peptide CS1 has the primary structure DELPQLVTL-PPHNLHGPEILDVPST and is present within the 33-kD heparin-binding fragment of human plasma fibronectin (McCarthy et al., 1988a). This peptide has been reported to promote cell adhesion and spreading and to promote neurite extension by dorsal root ganglion neurons (Humphries et al., 1987, 1988; Dufour et al., 1988). Peptide CS1 is located to the carboxy-terminal side of both RGDS and the carboxy-terminal heparin-binding domain of fibronectin. Further evidence suggests that yet another cell adhesion/spreading domain is located to the amino-terminal side of the RGDS sequence (Obara et al., 1988). While receptors for the heparin-binding cell adhesion promoting domains of fibronectin have yet to be identified, peptide CS1 has been shown to promote cell adhesion through an integrin-dependent mechanism. Anti- β 1 integrin antibodies have been reported to perturb cell adhesion to peptide CS1 (Dufour et al., 1988; Humphries et al., 1988), and recently T lymphocyte adhesion to this region of fibronectin has been demonstrated to involve an $\alpha_4\beta_1$ integrin which is RGD-independent (Wayner et al., 1989).

These studies further examined the role for peptide I in melanoma cell adhesion and spreading. The results support the contention that peptide I, located within the carboxy-terminal heparin binding region of fibronectin, represents a major heparin-binding determinant that promotes cell adhesion in an RGD-independent manner. Furthermore, the adhesion-promoting activity of this peptide and of peptide CS1 could be distinguished by several criteria, suggesting that the two peptides promote cell adhesion by distinct mechanisms.

Materials and Methods

Cell Culture

The cells used for these studies were the metastatic melanoma cell line K1735 M4, originally supplied as a generous gift from Dr. I. J. Fidler (Houston, TX). Cells were passaged in vitro in DMEM containing 10% calf serum by methods previously described (McCarthy et al., 1986, 1988a). The metastatic potential of this cell line has been previously confirmed by

our laboratory (McCarthy et al., 1988b). Care was taken to limit the number of in vitro passages to 10 to minimize phenotypic drift.

Protein and Fragment Isolation

Human plasma fibronectin was purified as a byproduct of Factor VIII production by sequential ion-exchange and gelatin affinity chromatography as described (McCarthy et al., 1986, 1988a). The 33-kD heparin binding fragment, which originates from A-chains of fibronectin, was purified from tryptic/catheptic digests of intact fibronectin using sequential gelatin, heparin, and antibody affinity columns as described previously (McCarthy et al., 1988a). Laminin and type IV collagen were purified from the murine Englebreth-Holm-Swarm tumor as described previously (Herbst et al., 1988). The purity of intact proteins and the 33-kD fragment of fibronectin was verified by SDS-PAGE and Coomassie brilliant blue staining.

Peptide Synthesis and Characterization

Peptides from fibronectin were synthesized at the Microchemical Facility of the University of Minnesota by Dr. Robert Wohlheuter using a peptide synthesizer (System 990; Beckman Instruments Co., Fullerton, CA). The procedures used were based on the Merrifield solid-phase system as described previously (Stewart and Young, 1984). Lyophilized crude peptides were purified by preparative reverse-phase HPLC on a C-18 column, using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. The purity and composition of the peptides was verified by HPLC analysis of hydrolysates prepared by treating the peptides under nitrogen in 6 N HCl overnight at 110°C. The peptides included heparin binding peptides I and II (McCarthy et al., 1988a), which have the sequences YEKPGSPPREVVPRPRGV and KNNQKSEPLIGRKKT, respectively. Peptide CS1, which has the sequence DELPQLVTLPPHNLHGPEILDVPST (Humphries et al., 1987) was also synthesized. The sequences shown use the single-letter amino acid code (K = lysine, R = arginine, H = histidine, E = glutamic acid, D = aspartic acid, Q = glutamine, N = asparagine, P = proline, G = glycine, S = serine, T = threonine, V = valine, I = isoleucine, L = leucine, Y = tyrosine).

Coupling of Synthetic Peptides to IgG

Peptides I and CS1 were coupled to normal rabbit IgG as suggested by Humphries et al. (1987). However, we used carbodiimide as a coupling reagent, based on a procedure described previously (Bauminger and Wilchek, 1980). Briefly, equal amounts (by weight) of peptide and IgG were solubilized in water and mixed with a 10-fold excess (by weight) of 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma Chemical Co., St. Louis, MO) dissolved in water. The sample was then mixed overnight at 4°C on a circular rotator. The coupled peptide was then dialyzed extensively in PBS to remove the excess carbodiimide and uncoupled peptide (10,000-D exclusion; Spectrum Medical Industries, Los Angeles, CA).

Cell Adhesion Assays

The cell adhesion assay was essentially the same as described previously (Skubitz et al., 1987). Briefly, purified fibronectin, 33-kD heparin-binding fragment of fibronectin, or the various synthetic peptides were diluted to various concentrations in PBS, and 50- μ l aliquots were dispensed in triplicate to microtiter wells (Immulon 1; Dynatech Laboratories, Chantilly, VA). The wells were dried overnight in a 29°C oven, and nonspecific sites on the plastic were then blocked for 3 h by treating the wells with 200 μ l of PBS containing 2 mg/ml fatty acid-free BSA (Miles Laboratories, Elkhart, IN). Alternatively, purified fibronectin, normal rabbit IgG, or the peptides that were coupled to normal rabbit IgG were diluted in PBS and 100- μ l aliquots were dispensed in triplicate into microtiter wells. The wells were incubated at room temperature for 60 min as described (Yamada and Kennedy, 1984; Humphries et al., 1987), nonadsorbed proteins were removed, and the wells were incubated with 2 mg/ml BSA in PBS for 3 h.

Subconfluent K1735 M4 melanoma cells, which had been radiolabeled overnight with tritiated thymidine, were released from culture flasks by trypsin/EDTA, washed, and adjusted to a concentration of 5×10^4 cells per ml of DME containing 0.15 mM Hepes (pH 7.2) and 2 mg/ml BSA. The cells were dispensed in a volume of 100 μ l to the microtiter wells. Depending on the experiment, the incubation time for the assay ranged from 30 min to 2 h. The assays were terminated by aspirating loosely bound and unbound cells from the wells, washing the wells three times, and solubilizing the bound cells in 0.5 N NaOH containing 1% SDS. Bound radioactivity, deter-

mined in a liquid scintillation counter (model 3801; Beckman Instruments Co.), was used to calculate the percentage of cells adherent in each sample. In parallel experiments, adherent cells were fixed with 2% glutaraldehyde in PBS and photographed on a Nikon Diaphot inverted-phase microscope.

Cell adhesion was inhibited both by synthetic peptides and polyclonal antibodies raised against the synthetic peptides. To test for inhibition of cell adhesion by synthetic peptides, cells were first preincubated in various concentrations of synthetic peptides for 30 min before the cell adhesion assay. The exogenous peptides were included in the adhesion assay for the duration of the experiment. When antibodies were tested for inhibition of cell adhesion, various concentrations of purified immunoglobulins generated against peptide I or normal rabbit immunoglobulin were diluted in the cell adhesion medium and preincubated on the plates for 30 min before the addition of cells. As was the case for the peptide inhibition experiments, the antibodies remained in the cell adhesion assay for the duration of the experiment. After the 30-min preincubation, the cells were incubated in the protein-coated wells for 30 min. Nonadherent cells were then aspirated off, the wells were washed, and bound cells were quantitated as described above.

Purification of Commercial [3 H]Heparin

Before use in the [3 H]heparin solid-phase binding assays, commercial [3 H]heparin (0.03 mCi/mg sp act; ICN Immunobiologicals, Lisle, IL) was first purified to create a sample that was homogeneous by size and charge. The [3 H]heparin was first applied to a Sephadex G-50 column (0.5 \times 25 cm) equilibrated in 0.1 M ammonium formate. The radioactivity in the void volume (V_0) of this column was concentrated by lyophilization and resuspended in the starting buffer (DEAE buffer) for the (DEAE) ion exchange column: 0.05 M Tris-HCl, pH 7.0, containing 6 M urea, 0.1 M NaCl, and 0.2% CHAPS. [3 H]Heparin was purified by HPLC (model 110 A; Beckman Instruments Co.) using a 7.5 \times 75 mm TSK DEAE 5PW anion-exchange column (Bio-Rad Laboratories, Richmond, CA). The sample was applied at a flow rate of 1 ml/min, and the column was washed with 20 column volumes of DEAE-buffer. [3 H]Heparin was eluted with a linear salt gradient from 0.1 M to 0.8 M NaCl over 45 min (a rate of 0.015 M/min). The radioactive peak from this column was dialyzed against water, concentrated by lyophilization, and resuspended in 0.5 M sodium acetate buffer containing 0.2% CHAPS. The hydrodynamic size of [3 H]heparin was estimated by gel filtration on 0.9 \times 110 cm Sepharose CL-6B columns. The columns were equilibrated and eluted with 0.5 M sodium acetate containing 0.2% CHAPS, pH 7.0, at a flow rate of 3 to 4 ml/h (Klein et al., 1986). Fractions of 1.0–1.2 ml were collected with a 95% recovery of radioactivity. Rat chondrosarcoma chondroitin sulfate proteoglycan (Oegema et al., 1975) associated with 4% hyaluronic acid, and glucuronolactone, were used to mark the column void (V_0) and total (V_t) volumes, respectively. [3 H]Heparin chain size was estimated based on the previous data of Wasteson (1971). The concentration of the purified [3 H]heparin was determined by uronic acid analysis as described previously (Bitter and Muir, 1964).

Solid-Phase [3 H]Heparin Binding Assay

The ability of the various ligands to bind purified [3 H]heparin was assessed using a solid-phase binding assay that used either Immulon 1 plates (Skubitz et al., 1988) or Immulon C plates (McCarthy et al., 1988a) as a support. In either case, the substrata were prepared by drying the various ligands onto the surface of the wells as for the cell adhesion assays. The plates were then blocked with PBS/BSA, and purified [3 H]heparin was added to the wells in PBS/BSA. The plates were incubated for 2 h at 37°C, at which time they were washed 5 times with PBS to remove the unbound [3 H]heparin. The bound [3 H]heparin was solubilized with 0.5 N NaOH containing 1% SDS, and the radioactivity in each sample was quantitated as described above.

In certain experiments, unlabeled heparin (molecular mass 15 kD, Sigma Chemical Co.), dextran sulfate (molecular mass 8 kD, Sigma Chemical Co.) or chondroitin-4 sulfate glycosaminoglycans from the rat Swarm chondrosarcoma (Oegema et al., 1975; molecular mass 20 kD, generously supplied by Dr. T. R. Oegema) were used in competition assays for [3 H]heparin binding to the various ligands. For these experiments, various concentrations of competitor were mixed in PBS/BSA with subsaturating levels of [3 H]heparin before addition to the assay, and the assay performed as described above.

Purified immunoglobulin generated against peptide I was also tested for the ability to inhibit [3 H]heparin binding to intact fibronectin. For these assays, various concentrations of purified anti-peptide I immunoglobulin or normal rabbit immunoglobulin were diluted in PBS/BSA and incubated on fibronectin coated plates at 37°C for 30 min before the assay. A subsaturat-

ing level of [3 H]heparin was then added to the wells, incubated, and quantitated as described above.

Generation and Purification of Polyclonal Antibodies against Peptide I

Polyclonal antibodies were generated against heparin binding peptide I coupled to keyhole limpet hemocyanin (KLH¹, Sigma Chemical Co.) using carbodiimide as a coupling reagent, as described above for coupling to normal rabbit IgG. However, after the coupling reaction, the coupled peptide was dialyzed in Spectrapore 6 tubing (Spectrum Medical Industries) with an 1,000-D exclusion so that only the excess carbodiimide was removed and any uncoupled peptide would remain in the tubing. This mixture was then concentrated in Aquacide II (Calbiochem-Behring Corp., La Jolla, CA) to a final concentration of 10 mg/ml, and aliquots were stored at -70°C. The coupling of the peptide to KLH was verified by the use of radioiodinated peptide in a parallel reaction mixture.

The coupled mixture was then used to immunize New Zealand White Rabbits. Immunization was performed by mixing an equal volume of peptide/KLH conjugate with CFA and injecting this mixture into multiple sites on the shaved backs of the rabbits (~2 mg of conjugate/rabbit). Subsequent biweekly boosts in incomplete Freund's adjuvant were injected i. m. into the hind legs of rabbits. Sera were collected 7–10 d after the sixth immunization, and tested by ELISA for reactivity against uncoupled peptide I and various other ligands.

IgG was purified from pooled immune sera by precipitation with a final concentration of 45% ammonium sulfate overnight at 4°C. The precipitate was pelleted by centrifugation, resolubilized, and dialyzed against 0.035 M NaCl in 0.025 M Tris, pH 8.2. The IgG was purified over a DEAE anion exchange column as described previously (Skubitz et al., 1988). Purity of the IgG was determined by SDS-PAGE and Coomassie brilliant blue staining of the gel. Retained immunoreactivity of the purified IgG was verified by ELISA.

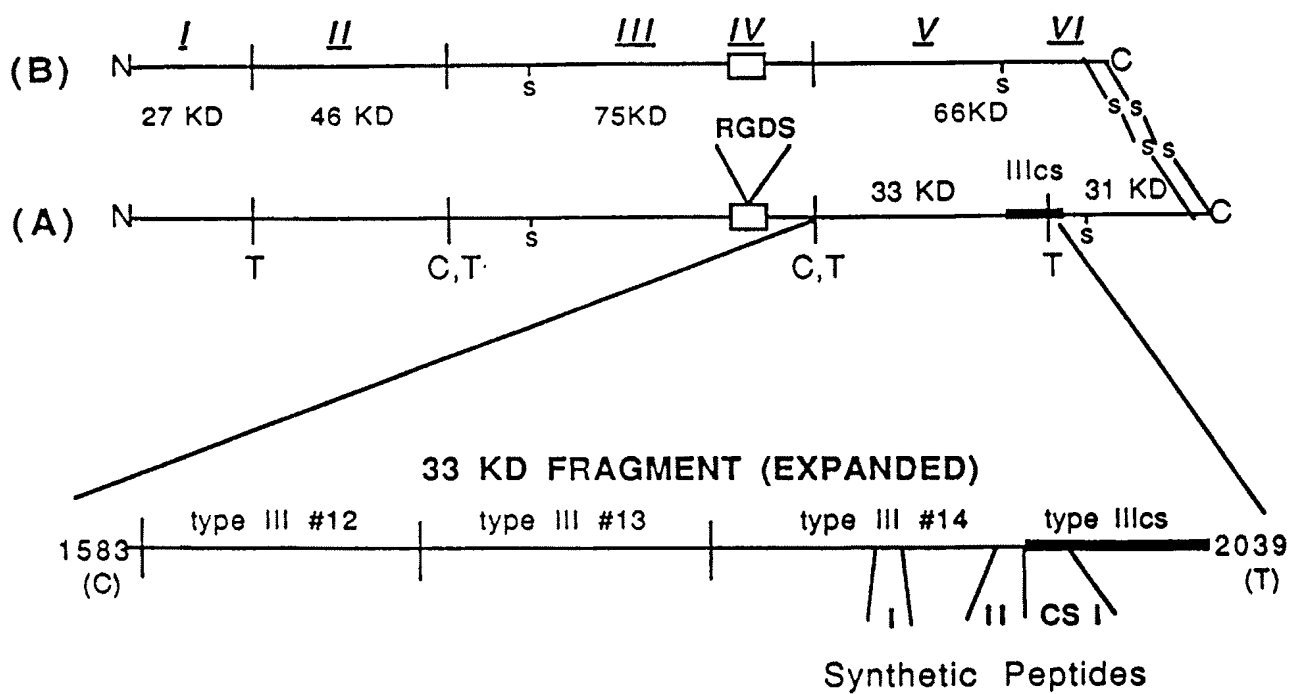
ELISA plates (either Immulon I or C) were prepared as for the cell adhesion assays by drying 1 μ g/well of the various ligands onto the plates, except Vollers buffer was used to dilute the ligands (Smith and Furcht, 1982). The plates were then blocked by incubating the wells for 2 h at room temperature with PBS containing 2 mg/ml of ovalbumin (PBS/OA). Various concentrations of purified IgG were diluted in PBS containing 0.05% Tween 20 and 0.36 M NaCl (PBS/Tween) and incubated on the plates for 1 h at room temperature on a rotating platform. The plates were washed three times with PBS/Tween and incubated an additional hour with a 1:500 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin (Cappel Laboratories, Malvern, PA). The plates were washed extensively with PBS/Tween and developed with the subsequent addition of 0.4 mg/ml of orthophenylene diamine containing 0.012% H₂O₂ in 0.1 M sodium phosphate and 24 mM citric acid. The reaction was terminated by the addition of 2.5 M H₂SO₄ and the intensity at 490 nm determined using an automatic microplate reader (model EL311; Bio-Tek Instruments, Burlington, VT).

Results

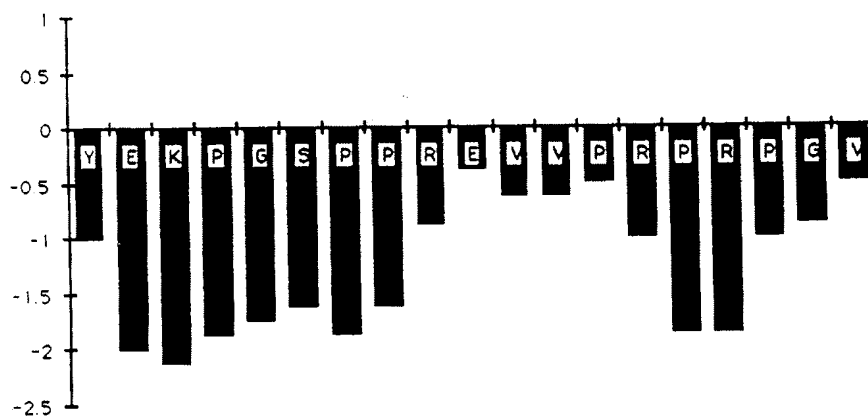
Peptides I and CS1 Promote RGD-independent Melanoma Cell Adhesion by Distinct Mechanisms

Synthetic peptides I and CS1 are clearly distinct in terms of primary structure, hydropathy profiles, and charge (Fig. 1). Peptide I, which is in type III repeat #14 of human plasma fibronectin (Fig. 1, Kornbliht et al., 1985), represents residues 1906–1924, and would be predicted to occur in all isoforms of fibronectin identified to date (Hynes, 1985). Peptide CS1 represents residues 1961–1985 and occurs only on a subset of human plasma fibronectin molecules (Kornbliht et al., 1985). The hydropathy values, which were calculated by the method of Kyte and Doolittle (1982), were determined by stepwise averaging across a window of 6 residues. According to this method, the more hydrophobic regions of the peptides correspond to the more positive numerical values. Overall,

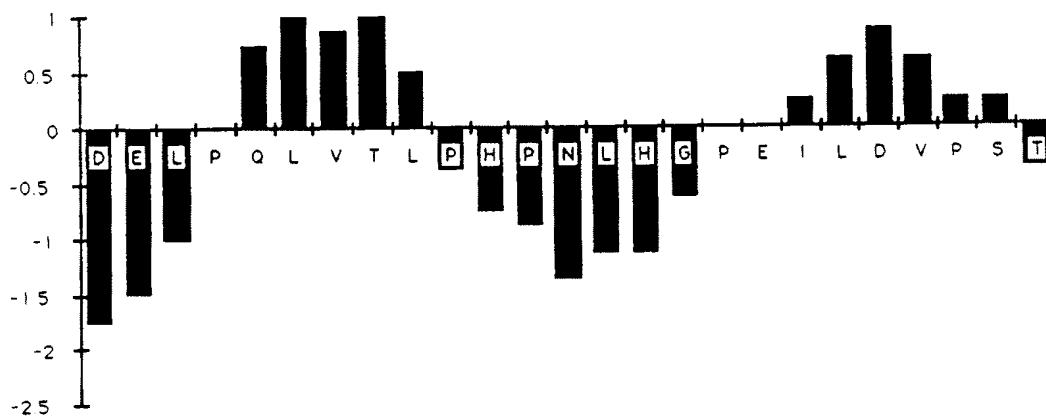
1. Abbreviations used in this paper: CS, chondroitin-4 sulfate; KLH, keyhole limpet hemocyanin; OA, ovalbumin.



PEPTIDE I



PEPTIDE CS.1



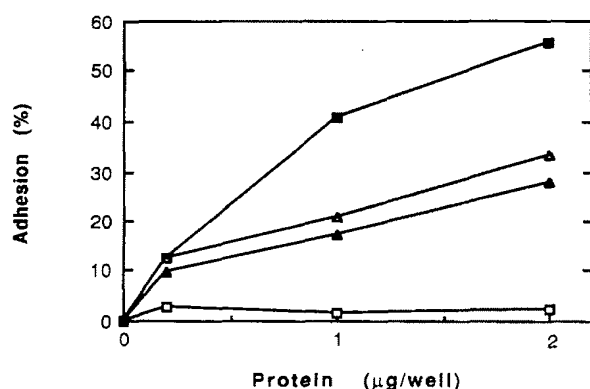


Figure 2. Adhesion of melanoma cells to increasing concentrations of synthetic peptides. Cells were radiolabeled and incubated for 2 h at 37°C in microtiter wells coated with various concentrations of fibronectin (■), peptide I (Δ), peptide CS1 (▲), or BSA (□) as described in Materials and Methods. Each value represents the mean of three separate determinations, and the SEM was <5% in each case. Three separate experiments gave similar results.

peptide CS1 is significantly more hydrophobic than peptide I (total net hydrophathy indices of -9.9 and -24.3, respectively), with two hydrophobic regions distributed on either side of a slightly hydrophilic region. In contrast, peptide I is amphipathic, containing two extremely hydrophilic regions which surround a relatively hydrophobic central portion of the peptide. Peptide CS1 also carries a net negative charge (-4) at neutral pH, in contrast to peptide I which is cationic in nature (+2 net charge).

Uncoupled peptides I and CS1, adsorbed directly to plates, promoted melanoma cell adhesion in a concentration-dependent manner (Fig. 2). The maximum level of adhesion (~35% of input cells) observed on peptide I (2 μg/well) was ~60% of that observed on intact fibronectin, and was virtually equivalent to cell adhesion levels on peptide CS1. Similar results were obtained when substrata were coated with peptides which had been coupled to IgG (data not shown).

Melanoma cell spreading also occurred on substrata coated with peptide I, regardless of whether or not the peptide had been coupled to IgG (compare Fig. 3, *a* and *b*); however, the rate of spreading on peptide I was somewhat slower than that observed on intact fibronectin (not shown). By contrast, the ability of cells to spread on substrata coated with peptide CS1 was directly dependent on whether or not the peptide had been coupled to IgG before coating. Substrata coated

with peptide CS1 coupled to IgG (Fig. 3 *c*) spread to the same extent as cells adherent on peptide I-coated substrata or substrata coated with intact fibronectin (Fig. 3 *e*). However, when substrata were coated with peptide CS1 that had not been coupled, very few cells exhibited a spread morphology (Fig. 3 *d*), despite the fact that such substrata could support the same level of cell adhesion as peptide I-coated substrata (Fig. 2). Virtually no adhesion and spreading of cells occurred on substrata coated with sham coupled IgG (Fig. 3 *f*) or BSA (not shown).

Our previous results (McCarthy et al., 1986, 1988*a*) as well as the results of others (Wayner et al., 1989) demonstrated that cell adhesion to the 33-kD fragment of fibronectin is independent of the RGDS cell adhesion sequence. Therefore, the effect of exogenous GRGDSP on melanoma cell adhesion to peptides I and CS1 was examined (Fig. 4). In agreement with our previous results, exogenous GRGDSP was virtually ineffective at inhibiting cell adhesion to the 33-kD fragment of fibronectin. However, GRGDSP was effective at inhibiting melanoma cell adhesion to intact fibronectin, with a maximum level of 50% inhibition observed in the presence of 600 μg/ml GRGDSP. Importantly, cell adhesion to peptides I and CS1 was unaffected by the addition of GRGDSP, even at concentrations which maximally inhibited cell adhesion to intact fibronectin (Fig. 4). These results indicate that the adhesion determinants within both synthetic peptides constitute major RGDS-independent cell adhesion sites within the 33-kD fragment of fibronectin. As controls in this study, GRGDSP was observed to have no effect on cell adhesion to laminin- or type IV collagen-coated substrata (data not shown).

Exogenous peptide I inhibited cell adhesion on substrata coated with fibronectin, peptide I, or the 33-kD fragment in a concentration-dependent manner (Fig. 5 *A*). The inhibition profiles on all three substrata were remarkably similar. However, the maximum level of inhibition on peptide I (~70%) was greater than the maximum inhibition observed on either the 33-kD fragment or intact fibronectin (~50%). CS1-mediated cell adhesion, in contrast, was totally resistant to competition with peptide I, even at a concentration of 600 μg/ml of exogenous peptide I. Exogenous peptide I also had no effect on melanoma cell adhesion to laminin or type IV collagen (data not shown).

Exogenous peptide CS1 inhibited cell adhesion to substrata coated with intact fibronectin, the 33-kD fragment, and peptide CS1 itself (Fig. 5 *B*). The inhibition profiles on substrata coated with intact fibronectin and the 33-kD fragment were similar, with maximum levels of inhibition at ~40%. Adhesion to peptide CS1 was most sensitive to the

Figure 1. Location of synthetic peptides within intact fibronectin and hydrophathy profiles of peptides I and CS1. Shown is a schematic diagram depicting the location of the 33-kD carboxy-terminal heparin binding fragment within fibronectin and the two synthetic heparin binding peptides (peptides I and II) as well as peptide CS1. In contrast to peptides I and II, which are present within a type III homology common to all isoforms of fibronectin, peptide CS1 is restricted to isoforms of human plasma fibronectin that contain the type IIIc region (A-chains). The amino terminal end and the carboxy-terminal limit of the 33-kD fragment is based on previous sequence data (McCarthy, 1988*a*). Selected biological domains, indicated by italicized, underlined roman numerals at the top of the figure, are based on the nomenclature of Furcht (1981). (I) Weak heparin binding; (II) collagen binding (noncovalent); (III) free sulfhydryl; (IV) RGD-mediated cell adhesion; (V) carboxy-terminal strong heparin binding and cell adhesion; (VI) free sulfhydryl. Approximate locations of tryptic (T) and cathepsin D (C) sites on intact fibronectin are shown. The sequences and hydrophathy profiles for peptides I and CS1 are shown in the lower portion of the figure. The hydrophathy profiles are calculated across a six-residue window and are based on the values of Kyte and Doolittle (1982). According to this method, the more positive values reflect increasingly hydrophobic character.

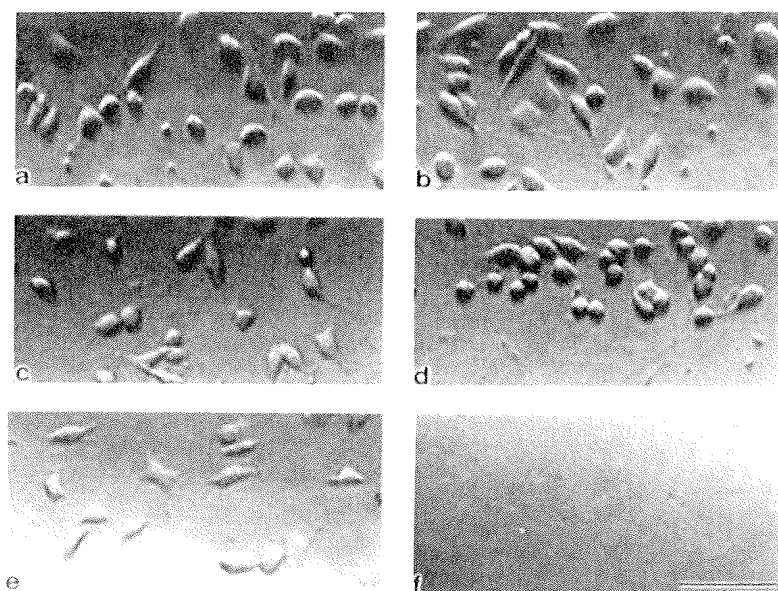


Figure 3. Spreading of melanoma cells on surfaces coated with synthetic peptides. Peptides I (a) and CS 1 (c) were coupled to IgG as described in Materials and Methods and diluted to a final concentration of 500 $\mu\text{g/ml}$ IgG. Wells were then coated with 100 μl of peptide:IgG conjugate or IgG alone (f). Alternatively, wells were coated with 1 μg of uncoupled peptide I (b), CS 1 (d), or intact fibronectin (e). Cells were then incubated for 2 h at 37°C in these microtiter wells and photographed as described in Materials and Methods. Bar in f, 45 μm . Two separate experiments gave similar results.

effects of soluble peptide CS1 (70% maximum). Importantly, peptide I-mediated cell adhesion was totally resistant to the effects of soluble peptide CS1 at all concentrations. Cell viability, as assessed by trypan blue dye exclusion after a 1-h incubation in peptides, was >90% in all of these assays.

Polyclonal IgG against Peptide I Specifically Inhibits Peptide I-mediated Melanoma Cell Adhesion

Polyclonal antibodies were generated in rabbits against pep-

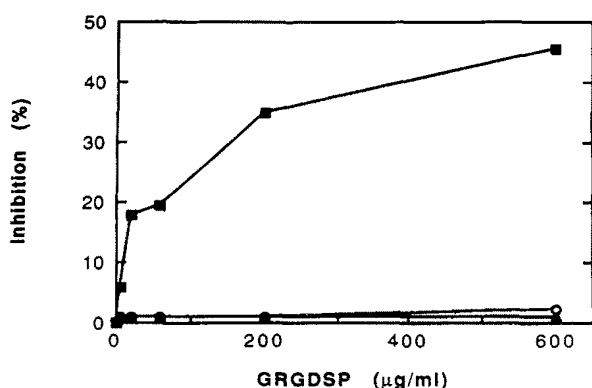


Figure 4. Peptide I- and peptide CS1-mediated cell adhesion is RGDS-independent. Cells were preincubated in the indicated concentrations of the hexapeptide GRGDSP for 30 min at 37°C and then added to wells coated with 1 μg fibronectin (■), or 2 μg of 33 kD fragment (○), peptide I (△), or peptide CS1 (▲). The assay was terminated 30 min later as described in Materials and Methods and the percentage of adherent cells was determined. The percent inhibition of cell adhesion was quantitated as: (1 minus [the number of cells bound in the presence of the inhibitor divided by the number of cells bound in the absence of the inhibitor]) multiplied by 100. Each value represents the mean of three separate determinations, and the SEM was <5% in each case. Three separate experiments gave similar results.

tide I coupled to KLH. Immune sera were collected and the purified IgG screened by ELISA for immunoreactivity against various ligands. Anti-peptide I IgG reacted very well with intact fibronectin, the 33-kD fragment, and peptide I (Fig. 6); however, as would be expected, the extent of reactivity was much greater towards peptide I than towards the 33-kD fragment or intact fibronectin. This IgG was quite specific because it did not react by ELISA against substrata coated with ovalbumin, peptide CS1, or fibronectin peptide II (see Fig. 1), which also binds [^3H]heparin and promotes melanoma cell adhesion (McCarthy et al., 1988a). In addition, the anti-peptide I IgG did not react with laminin, type IV collagen, or BSA (not shown).

The effect of anti-peptide I IgG on cell adhesion to various ligands was next determined (Fig. 7). Increasing concentrations of IgG were extremely effective at inhibiting melanoma cell adhesion to the 33-kD fragment, with a highly significant (70%) inhibition observed in the presence of 500 $\mu\text{g/ml}$ of anti-peptide I IgG. This IgG was even more effective at inhibiting cell adhesion to substrata coated with peptide I, with a virtually complete (95%) inhibition observed at the highest concentration of IgG. In contrast, anti-peptide I IgG was minimally effective (20–25%) at inhibiting adhesion to intact fibronectin, which would be expected since cells should be able to adhere to RGD-related and other adhesion sequences within fibronectin not recognized by the antibodies. Anti-peptide I IgG had no effect on cell adhesion to laminin, peptide CS1, or peptide II (not shown). Normal rabbit immunoglobulin had no effect on melanoma cell adhesion to any of the substrata tested (data not shown).

Peptide I (But Not CS1) Represents a Major Heparin-binding Determinant on Intact Fibronectin

The ability of peptides I and CS1 to bind [^3H]heparin were compared using a solid-phase binding assay in which the peptides were bound to the substrata. Before use in these assays, commercial [^3H]heparin was first purified to obtain a fraction that was relatively homogenous in size and charge

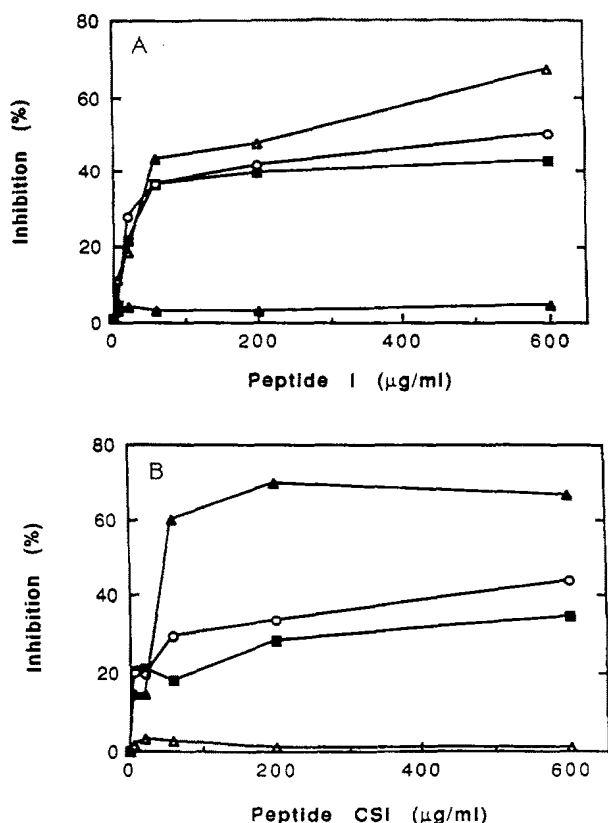


Figure 5. Effects of exogenous peptide I and peptide CSI on melanoma cell adhesion. Radiolabeled cells were preincubated for 30 min in increasing concentration of (A) peptide I or (B) peptide CSI, then added to wells coated with 1 μg of fibronectin (■), 2 μg 33-kD fragment (○), peptide I (Δ), or peptide CSI (▲). After a 30-min incubation, the nonadherent cells were aspirated off and the wells were washed three times. Adherent cells were quantitated in a scintillation counter. The percent inhibition of cell adhesion was quantitated as: (1 minus [the number of cells bound in the presence of the inhibitor] divided by the number of cells bound in the absence of the inhibitor) multiplied by 100. Each value represents the mean of three separate determinations, and the SEM was <10% in each case.

characteristics. The V_0 fractions of a Sephadex G-50 chromatographic separation of commercial [3 H]heparin (Fig. 8 A) were pooled, applied to an HPLC-DEAE anion exchange column and eluted as a single, sharp peak from this column at a salt concentration of 0.56 M NaCl (Fig. 8 B). This radioactive peak was pooled, concentrated, and applied to an analytical Sepharose CL-6B column from which it eluted at K_{av} 0.68 (Fig. 8 C). The procedure yielded [3 H]heparin with an estimated molecular weight of ~8 kD (according to the data of Wasteson, 1977) and a specific activity of 0.08 mCi/mg uronic acid.

Purified [3 H]heparin bound in a concentration dependent manner to substrata coated with fibronectin, the 33-kD fragment, or peptide I (Fig. 9). The binding of [3 H]heparin to each of these ligands was specific as a 100-fold excess of unlabeled heparin completely inhibited binding. In contrast, substrata coated with 5 μg/well of peptide CSI were totally

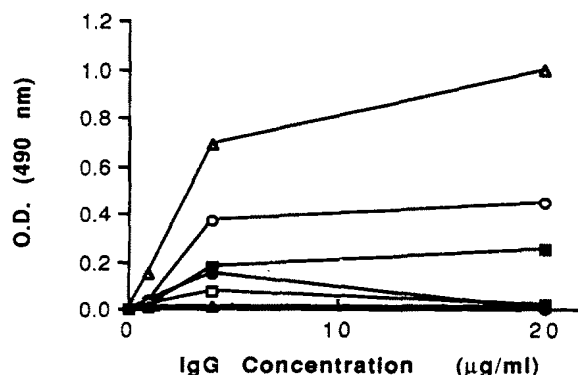


Figure 6. Reactivity of purified IgG raised against peptide I-KLH. Plates were coated with 1 μg of fibronectin (■), the 33-kD fragment (○), peptides I (Δ), II (●), CSI (▲), or OA (□). Purified IgG from rabbits immunized with peptide I coupled to KLH was then added to the wells at the indicated concentrations. The ELISA was then performed as described in Materials and Methods and absorbance readings at 490 nm were taken. The values shown represent the average of triplicate wells with background readings using normal rabbit IgG subtracted. Four experiments gave similar results.

ineffective at binding [3 H]heparin, despite the fact that such substrata could promote melanoma cell adhesion. Increasing the coating concentration of peptide CSI to levels as high as 10 μg/well failed to promote the binding of [3 H]heparin (data not shown).

The effect of various salt concentrations on the [3 H]heparin binding of fibronectin, the 33-kD fragment, and peptide I was compared (Table I). At physiological salt concentrations (0.15 M), each of the three ligands bound [3 H]heparin to es-

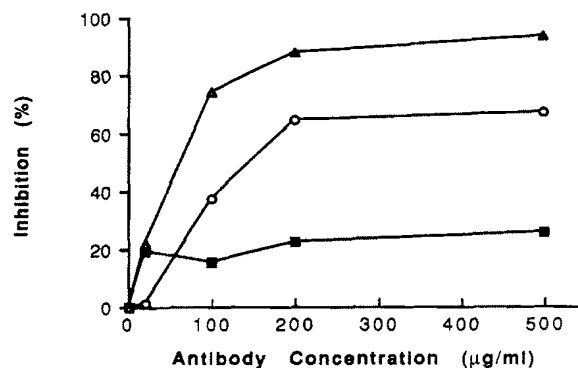


Figure 7. Effect of anti-peptide I antibodies on melanoma cell adhesion to intact fibronectin, 33 kD fragment, and peptide I. Radiolabeled cells were incubated for 30 min at 37°C in microtiter wells in the presence of increasing concentrations of purified IgG from rabbits immunized with peptide I as described in Materials and Methods. The wells were coated with 1 μg of fibronectin (■), 2 μg of 33-kD fragment (○), or peptide I (Δ). The percent inhibition of cell adhesion was quantitated as: (1 minus [the number of cells bound in the presence of the inhibitor] divided by the number of cells bound in the absence of the inhibitor) multiplied by 100. Inhibition of cell adhesion in the presence of normal rabbit IgG was then subtracted at the corresponding IgG concentration. Each value represents the mean of three separate determinations, and the SEM was <5% in each case. Three separate experiments gave similar results.

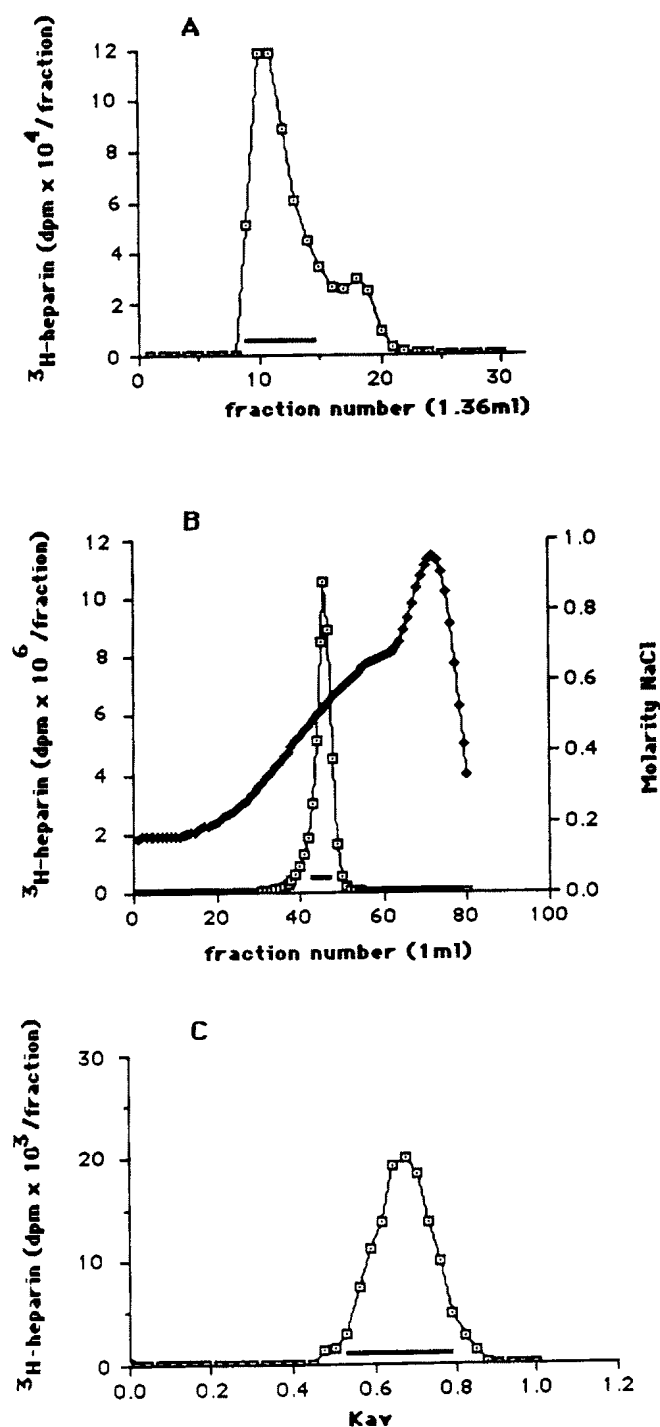


Figure 8. Purification of commercial $[^3\text{H}]$ heparin. (A) Commercial $[^3\text{H}]$ heparin was applied to and eluted from a Sephadex G-50 column as described in Materials and Methods. (B) The V_0 fractions (1.36 ml/fraction) were pooled as indicated by the bar, concentrated, and applied to an HPLC DEAE column equilibrated in 0.05 M Tris-HCL, pH 7.0, containing 6 M urea, 0.1 M NaCl, 0.2% CHAPS. The HPLC-DEAE column was eluted with the indicated gradient of NaCl (\diamond) and the radioactivity of each 1.0-ml fraction determined (\square). (C) The entire peak comprising fractions indicated by the bar was pooled, concentrated, and applied to an analytical Sepharose CL-6B column equilibrated in 0.5 M sodium acetate containing 0.2% CHAPS. The peak fractions were pooled as indicated by the bar and used in the $[^3\text{H}]$ heparin binding assays.

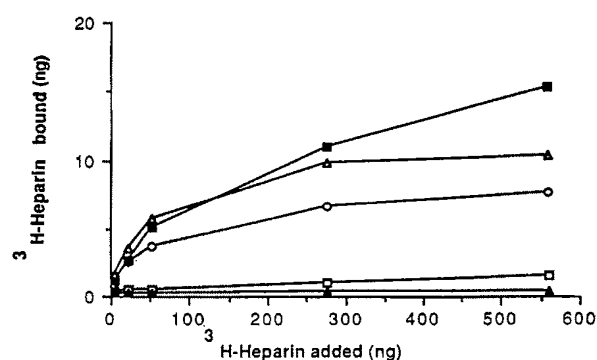


Figure 9. Binding of $[^3\text{H}]$ heparin to substrata coated with various ligands. Substrata were coated with 4 μg of intact fibronectin (\blacksquare), 10 μg of 33-kD fragment (\circ), 5 μg of peptides I (\blacktriangle) or CSI (\triangle), or 200 μg of BSA (\square) as described in Materials and Methods. The wells were then blocked with PBS/BSA, incubated for 2 h in the presence of the indicated concentrations of purified $[^3\text{H}]$ heparin, and the amount of bound $[^3\text{H}]$ heparin determined. The binding of $[^3\text{H}]$ heparin in the presence of a 100-fold excess of unlabeled heparin was at background levels of radioactivity. Each value represents the mean of three separate determinations, and the SEM was $<10\%$ of the mean values in each case.

essentially the same extent. The capacity of fibronectin to bind $[^3\text{H}]$ heparin was increased approximately twofold when the binding was performed in low (0.05 M) salt; however, no such increase was observed for the 33-kD fragment or peptide I. This is what would be expected if the amino terminal weak heparin binding domain of fibronectin (Smith and Furcht, 1982; McCarthy et al., 1986; see Fig. 1, domain I) exhibited activity at this lower salt concentration. Increasing the salt concentration above 0.15 M inhibited the binding of $[^3\text{H}]$ heparin to essentially the same extent on all three substrata. Minimal $[^3\text{H}]$ heparin binding to each of these three ligands was observed in the presence of 0.25 M NaCl, with essentially background binding observed at salt concentrations higher than 0.25 M NaCl.

The ability of related polysaccharides to inhibit the binding of $[^3\text{H}]$ heparin to intact fibronectin and peptide I was

Table 1. Effect of Salt on the Binding of $[^3\text{H}]$ Heparin to Fibronectin, 33-kD Fragment, or Peptide I

Substratum*	Heparin bound (ng) [†]			
	0.05 M	0.15 M	0.25 M	0.35 M
Intact fibronectin	6.3	3.7	0.3	0.2
33-kD fragment	3.7	3.0	0.6	0.3
Peptide I	3.4	3.0	0.7	0.2
BSA	0.4	0.7	0.3	0.2

* Immulon 1-coated substrata were prepared by drying down 4 μg of intact fibronectin, or peptide I, 200 μg of BSA, and 10 μg of 33-kD fragment as described in Materials and Methods.

[†] The effect of the indicated salt concentrations on the binding of purified $[^3\text{H}]$ heparin to each substratum was determined. The assay was performed using binding buffers that contained the indicated concentrations of NaCl, and each condition was washed with the same concentration of salt used in the assay before the determination of the bound $[^3\text{H}]$ heparin. The bound radioactivity at the end of the assay was solubilized and determined as described and used to calculate the amount of heparin bound to the various substrata. Each well contained ~ 50 ng of heparin at the beginning of the assay. Values represent the means of triplicate determinations, with the SEs of the means $<10\%$ of mean values in all cases.

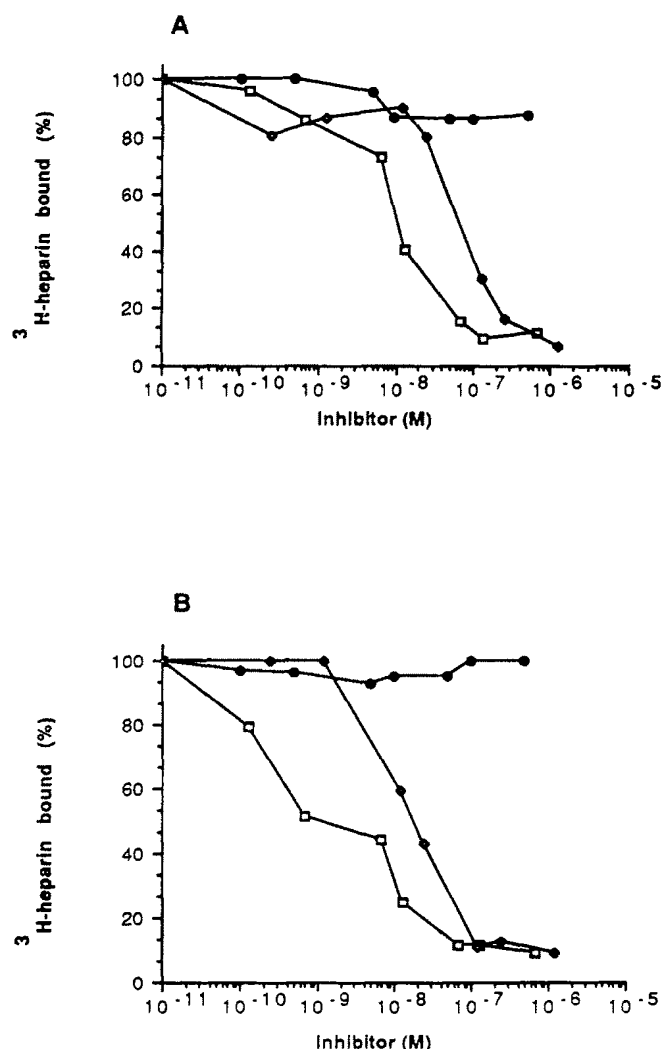


Figure 10. Inhibition of [³H]heparin binding to intact fibronectin and peptide I by sulfated polysaccharides. Substrata coated with intact fibronectin (A) or peptide I (B) were prepared as described in Materials and Methods. Subsaturation levels of [³H]heparin (50 ng/well) were mixed with the indicated concentrations of unlabeled heparin (□), dextran sulfate (◇), or chondroitin sulfate (●) and incubated in the wells for 2 h. The wells were then washed and the amount of bound [³H]heparin was determined. Each value represents the mean of three separate determinations, and the SEM was <10% in each case.

also compared (Fig. 10). Unlabeled heparin was most effective at inhibiting [³H]heparin binding to either substratum, producing a 50% inhibition at between 10⁻⁹ M (peptide I) and 10⁻⁸ M (fibronectin) competing heparin. Dextran sulfate was an order of magnitude less effective at inhibiting 50% of the binding of [³H]heparin to either substratum. At high concentrations, however, dextran sulfate could virtually eliminate heparin binding to either ligand, suggesting that high concentrations of dextran sulfate could nonspecifically inhibit [³H]heparin binding on the basis of charge. In contrast, less charged chondroitin-4 sulfate glycosaminoglycans were virtually ineffective at inhibiting the binding of [³H]-

heparin to peptide I or intact fibronectin, even at micromolar concentrations.

Finally, the ability of anti-peptide I IgG to inhibit the binding of [³H]heparin to intact fibronectin was examined (Fig. 11). This IgG inhibited the binding of [³H]heparin to intact fibronectin, in a concentration-dependent fashion. The highest concentration of IgG tested (500 µg/ml) inhibited ~60% of the [³H]heparin binding to intact fibronectin. In contrast, normal rabbit immunoglobulin was much less effective at inhibiting [³H]heparin (19% inhibition at 500 µg/ml, not shown).

Discussion

Our previous results were the initial ones that demonstrated that fibronectin contains RGDS-dependent and independent adhesion promoting activities (McCarthy et al., 1986, 1988a). A major RGDS-independent cell adhesion-promoting activity was first identified within an A-chain derived, carboxy-terminal 33-kD heparin-binding fragment of fibronectin (McCarthy et al., 1986) and others have observed similar results using a variety of normal and transformed cell types (Rogers et al., 1987; Mugnai et al., 1988; Wayner et al., 1989). These studies demonstrate that this fragment contains two cell adhesion-promoting domains that are localized within heparin binding peptide I (McCarthy et al., 1988a) and peptide CSI (Humphries et al., 1987). Although peptides I and CSI are both present within the 33-kD fragment (McCarthy et al., 1988a), the melanoma cell adhesion-promoting activity of these two synthetic peptides was shown to be distinct. First, cell adhesion to either peptide could not be cross-inhibited by the other peptide. Also, in contrast to peptide I, peptide CSI failed to bind [³H]heparin in a solid-phase binding assay. These results strongly suggest that peptides I and CSI interact with distinct cell surface receptors, although interaction of both peptides with distinct sites on a common receptor cannot be ruled out.

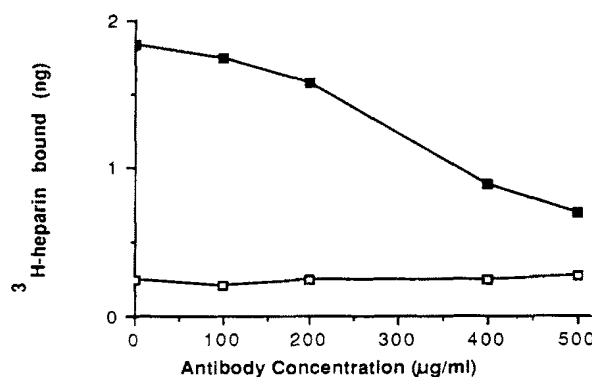


Figure 11. Effect of anti-peptide I IgG on [³H]heparin binding to intact fibronectin. Substrata coated with 5 µg of intact fibronectin (■) or 200 µg of BSA (□) were preincubated for 30 min in the presence of the indicated concentrations of purified anti-peptide I antibodies in PBS/BSA. Each well then received subsaturating amounts of [³H]heparin (~50 ng/well) and the plates were incubated for 2 h at 37°C. The wells were then washed and the amount of bound [³H]heparin was determined. Each value represents the mean of three separate determinations, and the SEM was <10% in each case.

An additional distinction between peptides I and CS1 was the requirement (or not) for the peptides to be coupled to a larger carrier protein to express the full complement of cell adhesion- and spreading promoting activities. Whereas peptide I was fully active with respect to both criteria regardless of whether or not it was coupled, peptide CS1 required coupling to a larger carrier molecule in order to exhibit spreading activity. Such a requirement for coupling of this peptide has been previously noted by Humphries et al. (1986) in the original description of this peptide. Despite the requirement of coupling for spreading, uncoupled peptide CS1 was active at promoting cell adhesion on coated substrata. Whereas this difference could indicate the presence of separate adhesion and spreading promoting determinants on peptide CS1, which is relatively large, it is more likely that the difference represents changes in the conformation (and hence specific activity) of the peptide that has been bound by the two different means.

The observation that the cell adhesion-promoting activity exhibited by peptide I was active within the 33-kD fragment of fibronectin was supported by two independent lines of evidence. First, exogenous peptide I could inhibit cell adhesion on the 33-kD fragment of fibronectin although the inhibition achieved with this peptide was never complete. Secondly, antibodies generated against peptide I bound directly to the 33-kD fragment of fibronectin (by ELISA) and were effective at inhibiting cell adhesion to the 33-kD fragment of fibronectin. Whereas these antibodies could virtually eliminate cell adhesion to peptide I, the inhibitory effects on the 33-kD fragment were never complete, consistent with the hypothesis that multiple distinct cell adhesion promoting determinants exist on the 33-kD fragment. Interestingly, anti-peptide I antibodies had a limited effect on cell adhesion to intact fibronectin. This is consistent with our previous results (McCarthy et al., 1988a) and the results of others (Woods et al., 1986), which demonstrated that antibodies that inhibit cell adhesion to heparin binding fragments of fibronectin have a limited effect on the level of cell adhesion to intact fibronectin. However, such antibodies have been demonstrated to potentiate the effects of exogenous RGD-containing peptides (McCarthy et al., 1988a) or to disrupt focal adhesion formation by normal fibroblasts (Woods et al., 1986).

Anti-peptide I antibodies, which reacted positively by ELISA against intact fibronectin or the 33-kD fragment, were also extremely (60%) effective at inhibiting [³H]heparin binding to intact fibronectin, indicating that peptide I represents a major heparin binding determinant on the carboxy-terminal heparin binding domain of intact fibronectin. The failure of these antibodies to completely inhibit heparin binding to intact fibronectin could relate, in part, to the contribution of other heparin binding determinants within this region of fibronectin (e.g., peptide II, which was not recognized by these antibodies; McCarthy et al., 1988a). This result further suggests that heparin binding to the carboxy-terminal region of fibronectin involves noncontiguous determinants within this region of the molecule. Multiple heparin-binding determinants have previously been proposed on other heparin binding proteins such as apolipoprotein B and vitronectin (Suzuki et al., 1985; Hirose et al., 1987; Cardin et al., 1989).

Peptide I was not as active on a molar basis as intact fibronectin at binding [³H]heparin, requiring a 100-fold

molar excess coating concentration compared with that used for fibronectin in order to achieve the same level of [³H]heparin binding. Despite this difference in absolute activities, a relative comparison of the heparin binding activities of peptide I and intact fibronectin showed remarkable similarity between these two ligands. The heparin binding activities of these two ligands (and the 33-kD fragment) were equally susceptible to the effect of progressively increasing salt concentrations (at physiologic levels or above). Similarly, the 50% inhibitory dose of dextran sulfate was much less effective (one to two orders of magnitude) than unlabeled heparin at inhibiting [³H]heparin binding to either of these two ligands. Chondroitin-4 sulfate (CS) was virtually ineffective in this regard, even at CS concentrations which were four orders of magnitude higher than the level of heparin that produced a 50% inhibition. Such specificity has previously been observed for heparin binding peptides from laminin (Charonis et al., 1988) and type IV collagen (Koliakos et al., 1989), although not all heparin-binding ligands show this same preference (Koliakos et al., 1989). This suggests, along with the antibody data, that the structural features of peptide I that are important for heparin-binding activity closely resemble those features of this sequence on intact fibronectin.

That peptide I-mediated cell adhesion could be distinguished from peptide CS1-mediated cell adhesion by several criteria suggests that the two peptides may mediate cell adhesion by interacting with distinct cell surface receptors, both of which are distinct from the well characterized RGD-dependent integrin interaction. Recent evidence has been presented which demonstrates that anti- β 1 integrin antibodies can inhibit CS1-mediated adhesion of neural crest cells or peripheral neurons, indicating that CS1-mediated adhesion may involve cell surface integrins (Dufour et al., 1988; Humphries et al., 1988; Wayner et al., 1989), which are interacting by an RGD-independent mechanism. By contrast, the heparin-binding activity of peptide I argues strongly for a role of cell surface proteoglycans/glycosaminoglycans in the recognition of the active sequence(s) within this peptide. That peptide I promotes cell spreading as well as adhesion indicates that the melanoma cell surface receptor for this sequence interacts either directly or indirectly with the cellular cytoskeleton of these cells. Cell surface heparan sulfate and chondroitin sulfate proteoglycans have been identified with properties consistent with a cell surface localization, which include an ability to bind to hydrophobic affinity columns or to become inserted into liposomes (Kjellén et al., 1981; Woods et al., 1985; Lories et al., 1987; David et al., 1989). Putative transmembrane sequences have also been identified on the core proteins of several proteoglycans (Sant et al., 1984; Noonan et al., 1988; Saunders et al., 1989), suggesting that such macromolecules could be involved in peptide I-mediated melanoma cell adhesion. It will clearly be of interest to use peptide I to determine the role of cell surface-associated proteoglycans in mediating melanoma cell adhesion to this region of fibronectin.

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References

- Bauminger, S., and M. Wilchek. 1980. The use of carbodiimides in the preparation of immunizing conjugates. *Methods Enzymol.* 70:151-159.
- Bitter, T., and H. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:466-468.
- Buck, C. A., and A. F. Horwitz. 1987. Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* 3:179-205.
- Cardin, A. D., and H. J. R. Weintraub. 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis.* 9:21-32.
- Charonis, A. S., A. P. N. Skubitz, G. G. Koliakos, L. A. Reger, J. Dege, A. M. Vogel, R. Wohlheuter, and L. T. Furcht. 1988. A novel synthetic peptide from the B1 chain of laminin with heparin binding and cell adhesion-promoting activities. *J. Cell Biol.* 107:1253-1260.
- David, G., V. Lories, A. Heremans, V. Der Schueren, J. J. Cassiman, and H. Van Den Berghe. 1989. Membrane-associated chondroitin sulfate proteoglycan of human lung fibroblasts. *J. Cell Biol.* 108:1165-1175.
- Dufour, S., J.-L. Duband, M. J. Humphries, M. Obara, K. M. Yamada, and J. P. Thiery. 1988. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple sites on fibronectin molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2661-2671.
- Furcht, L. T. 1981. Structure and function of the adhesive glycoprotein fibronectin. *Mod. Cell Biol.* 53-117.
- Herbst, T. J., J. B. McCarthy, E. Tsilibary, and L. T. Furcht. 1988. Differential effects of laminin, type IV collagen and specific domains of type IV collagen on endothelial cell adhesion and migration. *J. Cell Biol.* 106:1365-1373.
- Hirose, N., D. T. Blankenship, M. A. Krivanek, R. L. Jackson, and A. D. Cardin. 1987. Isolation and characterization of four heparin-binding cyanogen bromide peptides of human plasma apolipoprotein B. *Biochemistry.* 26:5505-5512.
- Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1987. Identification of two distinct regions of the type IIIc connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. Biol. Chem.* 262:6886-6892.
- Humphries, M., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1988. Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. *J. Cell Biol.* 106:1289-1297.
- Hynes, R. O. 1985. Molecular biology of fibronectins. *Annu. Rev. Cell Biol.* 1:67-90.
- Hynes, R. O. 1987. Integrin: A family of cell surface receptors. *Cell.* 48:549-554.
- Juliano, R. L. 1987. Membrane receptors for extracellular matrix molecules: relationship to cell adhesion and tumor metastasis. *Biochim. Biophys. Acta.* 907:261-278.
- Kjellén, L., I. Pettersson, and M. Höök. 1981. Cell-surface heparan sulfate: an intercalated membrane proteoglycan. *Proc. Natl. Acad. Sci. USA.* 78:5371-5375.
- Klein, D. M., D. M. Brown, and T. R. Oegema. 1986. Partial characterization of heparan and dermatan sulfate proteoglycans synthesized by normal rat glomeruli. *J. Biol. Chem.* 261:16636-16652.
- Koliakos, G. G., K. Kouzi-Koliakos, L. T. Furcht, L. A. Reger, and E. C. Tsilibary. 1989. The binding of heparin to type IV collagen: domain specificity with identification of peptide sequences from the $\alpha 1(IV)$ and $\alpha 2(IV)$ which preferentially bind heparin. *J. Biol. Chem.* 264:2313-2323.
- Kornbliht, A. R., K. Umezawa, K. Vibe-Pedersen, and F. E. Bareille. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755-1759.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.
- Liao, N. S., J. St. John, J. B. McCarthy, L. T. Furcht, and H. T. Cheung. 1989. Adhesion of lymphoid cells to the carboxyl terminal heparin-binding domains of fibronectin. *Exp. Cell Res.* 181:348-361.
- Liotta, L. A., C. V. Rao, and S. H. Barsky. 1983. Tumor invasion and the extracellular matrix. *Lab. Invest.* 49:636-649.
- Lories, V., H. De Boeck, G. David, J. J. Cassiman, and H. Van den Berghe. 1987. Heparan sulfate proteoglycans of human lung fibroblasts. Structural heterogeneity of the core proteins of the hydrophobic cell-associated forms. *J. Biol. Chem.* 262:854-859.
- McCarthy, J. B., M. L. Basara, S. L. Palm, D. F. Sas, and L. T. Furcht. 1985. The role of cell adhesion proteins, laminin and fibronectin, in the movement of malignant and metastatic cells. *Cancer Metastasis Rev.* 4:125-152.
- McCarthy, J. B., S. T. Hagen, and L. T. Furcht. 1986. Human fibronectin contains distinct adhesion and motility promoting domains for metastatic melanoma cells. *J. Cell Biol.* 102:179-188.
- McCarthy, J. B., M. K. Chelberg, D. J. Mickelson, and L. T. Furcht. 1988a. Localization and chemical synthesis of fibronectin peptides with melanoma adhesion and heparin binding activities. *Biochemistry.* 27:1380-1388.
- McCarthy, J. B., A. P. N. Skubitz, S. L. Palm, and L. T. Furcht. 1988b. Metastasis inhibition of different tumor types by purified laminin fragments and a heparin binding fragment of fibronectin. *J. Natl. Cancer Inst.* 80:108-116.
- Mugnai, G., K. Lewandowska, B. Carnemolla, L. Zardi, and L. A. Culp. 1988. Modulation of matrix adhesive responses of human neuroblastoma cells by neighboring sequences in the fibronectins. *J. Cell Biol.* 106:931-43.
- Noonan, D. M., D. A. Horigan, S. R. Ledbetter, G. Vogeli, M. Sasaki, Y. Yamada, and J. R. Hassell. 1988. Identification of cDNA clones encoding different domains of the basement membrane heparan sulfate proteoglycan. *J. Biol. Chem.* 263:16379-16387.
- Obara, M., M. S. Kang, and Yamada, K. M. 1988. Site directed mutagenesis of the cell binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. *Cell.* 53:649-679.
- Oegema, T. R., V. C. Hascall, and D. D. Dziewatowski. 1975. Isolation and characterization of proteoglycans from the Swarm rat chondrosarcoma. *J. Biol. Chem.* 250:6151-6159.
- Rogers, S. L., P. C. Letourneau, B. A. Peterson, L. T. Furcht, and J. B. McCarthy. 1987. Selective interaction of peripheral and central nervous system cells with two distinct cell-binding domains of fibronectin. *J. Cell Biol.* 105:1435-1442.
- Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57:375-413.
- Sant, A. J., S. E. Cullen, and B. D. Schwartz. 1984. Identification of a sulfate-bearing molecule associated with HLA class II antigens. *Proc. Natl. Acad. Sci. USA.* 81:1534-1538.
- Saunders, S., M. Jalkanen, S. O'Farrell, and M. Bernfield. 1989. Molecular cloning of syndecan, an integral membrane proteoglycan. *J. Cell Biol.* 108:1547-1556.
- Skubitz, A. P. N., A. S. Charonis, E. C. Tsilibary, and L. T. Furcht. 1987. Localization of a tumor cell adhesion domain of laminin by a monoclonal antibody. *Exp. Cell Res.* 173:349-360.
- Skubitz, A. P. N., J. B. McCarthy, A. S. Charonis, and L. T. Furcht. 1988. Localization of three distinct heparin binding domains of laminin by monoclonal antibodies. *J. Biol. Chem.* 263:4861-4868.
- Smith, D. E., and L. T. Furcht. 1982. Localization of two unique heparin binding domains of human plasma fibronectin with monoclonal antibodies. *J. Biol. Chem.* 257:6518-6523.
- Stewart, J. M., and J. D. Young. 1984. Solid Phase Peptide Synthesis. 2nd ed. Pierce Chemical Co. Rockford, IL.
- Suzuki, S., A. Oldberg, E. G. Hayman, M. D. Pierschbacher, and E. Ruoslahti. 1985. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2519-2524.
- Visser, M. R., G. M. Vercellotti, J. B. McCarthy, J. L. Goodman, T. J. Herbst, L. T. Furcht, and H. S. Jacob. 1989. Herpes Simplex virus inhibits endothelial cell attachment and migration to extracellular matrix proteins. *Am. J. Pathol.* 134:223-230.
- Wasteson, Å. 1971. A method for the determination of the molecular weight and molecular-weight distribution of chondroitin sulfate. *J. Chromatogr.* 59:87-97.
- Wayner, E. A., A. Garcia-Pardo, M. J. Humphries, J. A. McDonald and W. G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* 109:1321-1330.
- Woods, A., J. R. Couchman, S. Johansson, and M. Höök. 1986. Adhesion and cytoskeletal organization of fibroblasts in response to fibronectin fragments. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:665-670.
- Woods, A., J. R. Couchman, and M. Höök. 1985. Heparan sulfate proteoglycans of rat embryo fibroblasts: A hydrophobic form may link cytoskeleton and matrix components. *J. Biol. Chem.* 260:10872-10879.
- Yamada, K. M., and D. W. Kennedy. 1984. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. *J. Cell Biol.* 99:29-36.